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Significant differences in T cell receptor repertoires in lung adenocarcinomas with and without epidermal growth factor receptor mutations

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Recent clinical trials of non-small cell lung cancer with immune checkpoint inhibitors revealed that patients with epidermal growth factor receptor (*EGFR*) mutations had more unfavorable outcomes compared with those with wild-type *EGFR*. However, the underlying mechanism for the link between *EGFR* mutations and immune resistance remains unclear. We performed T cell receptor (TCR) repertoire analysis of resected lung adenocarcinoma tissues with and without *EGFR* mutations to investigate the characteristics of TCR repertoires. We collected a total of 39 paired (normal and tumor) lung tissue samples (20 had *EGFR* mutations) and conducted TCR repertoire analysis as well as whole-exome sequencing (WES) and transcriptome analysis. The TCR diversity index in *EGFR*-mutant tumors was significantly higher than that in *EGFR*-wild-type tumors (median [range] 552 [162-1,135] vs 230 [30-764]; $P < .01$), suggesting higher T cell clonal expansion in *EGFR*-wild-type tumors than in *EGFR*-mutant tumors. In WES, *EGFR*-mutant tumors showed lower numbers of non-synonymous mutations and predicted neoantigens than *EGFR*-wild-type tumors ($P < .01$, $P = .03$, respectively). The number of non-synonymous mutations revealed a positive correlation with the sum of frequencies of the TCRβ clonotypes of 1% or higher in tumors ($r = .52$, $P = .04$). The present study demonstrates significant differences in TCR repertoires and the number of predicted neoantigens between *EGFR*-mutant and wild-type lung tumors. Our findings provide important information for

Abbreviations: HLA, human leukocyte antigen; ICI, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1; RNA, ribonucleic acid; TCR, T cell receptor; WES, whole-exome sequencing

understanding the molecular mechanism behind *EGFR*-mutant patients showing unfavorable responses to immune checkpoint inhibitors.

KEYWORDS
EGFR mutation, lung adenocarcinoma, neoantigen, non-synonymous mutation, T cell receptor repertoire

1 | INTRODUCTION

Lung cancer is the most common cause of cancer-related death worldwide.[1] Despite advances in treatment modalities, such as combination chemotherapy and molecular-targeted therapy, over the past few decades the survival benefit has been restricted to a subset of patients with advanced diseases. New treatment modalities are urgently needed to target and eliminate invading tumor cells.

Recently, therapeutic antibodies that block the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway have induced robust and durable clinical responses in patients with various cancers, including advanced non-small cell lung cancer (NSCLC).[2,3] However, clinical benefits have been observed only in a small subset of patients, with response rates of approximately 20%-40% for advanced NSCLC.[2-5] In particular, retrospective analyses of clinical trials with PD-1/PD-L1 blockade, in which patients with NSCLC harboring epidermal growth factor receptor (EGFR) mutations were enrolled in clinical trials, demonstrated that patients with *EGFR*-mutant tumors responded more poorly to immune checkpoint inhibitors (ICI) compared to those with wild-type *EGFR*.[6-8] However, the molecular mechanism underlying the lower response rates to ICI remains unclear. Thus, elucidating the mechanism that cause the differences in the clinical responses to ICI between *EGFR*-mutation-positive and *EGFR*-mutation-negative groups is important to further improve outcomes and to optimize the use of these agents in lung cancer patients.

The progress in analyzing T cell receptor (TCR) repertoires in cancer tissues made it possible to evaluate the diversity of T cell clonotypes and the extent of clonal T cell expansion, and to characterize neoantigen-specific TCR.[9] Detailed information on the tumor microenvironment may serve as a predictive marker for immunomodulatory therapies and may also be useful for development of new treatment strategies, including personalized T cell-mediated cancer immunotherapy and neoantigen vaccine therapy.[10-14] TCR repertoire analyses could be used to monitor the dynamics of T cell clonality and the individual tumor-reactive T cell clones in cancer patients treated with ICI.[15,16] Profiling the immune repertoire by quantifying the TCR composition in tumor tissues enables assessment of T cell diversity and immune-related characteristics. Therefore, in the present study, we aimed to investigate the differences in immune-related conditions in *EGFR*-mutant/wild-type tumors using TCR sequencing along with whole-exome sequencing (WES) analysis.

Here, we report distinct characteristics of TCR repertoire patterns in lung adenocarcinomas with and without *EGFR* mutations and demonstrate the association between the diversity of TCR repertoires and the numbers of non-synonymous mutations in tumors. Our results should contribute to a better understanding of the molecular mechanism behind *EGFR*-mutant patients having shown an unfavorable response to ICI.

2 | MATERIALS AND METHODS

2.1 | Patients

All subjects in the present study received curative surgery between 2014 and 2017 at the Tohoku University Hospital. A total of 39 patients were enrolled based on the following inclusion criteria: (i) pathologically diagnosed with lung adenocarcinoma; (ii) pathological stage was I to III; (iii) frozen tumor and normal paired tissue samples were available; and (iv) written informed consent was obtained. This study was approved by the Institutional Review Board of Tohoku University (Sendai, Japan: approval no. 2013-1-592) and the University of Chicago (Chicago, IL, USA: approval no. 13-0797).

2.2 | Tissue samples

Tumor and adjacent normal lung tissue samples were obtained from the resected tissues. These resected tissue samples were immediately soaked in RNA later Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80°C until extraction of nucleic acids. All the samples were histologically confirmed as adenocarcinoma by pathologists.

2.3 | T cell receptor sequencing

We performed TCR sequencing as described previously.[17,18] Briefly, we synthesized complementary DNAs (cDNAs) with the common 5′-RACE adapter sequence, and amplified TCR β-chain (TCRβ) by PCR. We added Illumina index sequences with barcodes using the Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA), which allows barcode tagging and pooling of multiple samples. Subsequently, the prepared library was sequenced using the MiSeq Reagent Kit v3 (600-cycle) on the MiSeq System (Illumina). We calculated the diversity index (inverse Simpson's index) to quantify the clonality of the TCRβ repertoires, as previously described.[19]

2.4 | Whole-exome sequencing and data analysis

DNA and RNA were isolated using the standard procedures and the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). Whole-exome libraries were prepared from genomic DNA using the SureSelect XT Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) and analyzed as previously described.[18,20] Human leukocyte antigen (HLA) class I genotypes were determined using the WES data of normal tissues and the OptiType algorithm.[21] Neoantigens were predicted for each non-synonymous variant by defining all 8-mer to 11-mer peptides resulting from the mutation and determining the predicted binding affinity to HLA-A, B and C of <500 nM, using NetMHCv3.4 and NetMHCpanv2.8 software.[11,22-24]

2.5 | TCGA dataset analysis

We obtained the TCGA dataset from a previous publication.[25] Among these, 467 patients from the TCGA lung adenocarcinoma cohort had information of non-synonymous mutations as well as the *EGFR* status.

2.6 | Gene expression analysis

Gene expression quantitative PCR in tumor tissues was performed using the TaqMan gene expression assays (Thermo Fisher Scientific) on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). mRNA expression levels of *PD-1* (assay ID, Hs01550088_m1), *PD-L1* (CD274; assay ID, Hs01125301_m1), *CD8* (assay ID, Hs002335520_m1) and *FOXP3* (assay ID, Hs01085834_m1) were evaluated and normalized to *GAPDH* expression (assay ID, Hs02758991_g1).

2.7 | Statistical methods

Student's *t* test and Fisher's exact test were performed for comparison between tumors with and without *EGFR* mutations. The Mann-Whitney *U* test was used for comparison of the numbers of non-synonymous mutations, the TCRβ diversity index, the proportions of expanded TCRβ clonotypes, and the numbers of predicted neoantigens between tumors with and without *EGFR* mutations. Multiple logistic regression models were applied to assess the association between the *EGFR* mutation status and the binary measures of patient characteristics, including the diversity index. Pearson correlation (*r*) was used to analyze the association between the proportion of TCRβ clones and non-synonymous mutations. Statistical analysis was carried out using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). A *P*-value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | Patients

The characteristics of patients are summarized in Table 1. The median age of these patients was 68 years old (range: 41-85) and 25 patients (64%) were women. Nineteen patients (49%) were never-smokers, and pathological stages after curative lung resection were I for 9 patients (23%), II for 16 patients (41%) and III for 14 patients (36%). Tumors from 20 patients (51%) had *EGFR* mutations (an exon 19 deletion in 9 patients and an L858R mutation in 11 patients); 80% of these patients were women and 70% of them were never-smokers.

3.2 | T cell receptor repertoire analysis

To elucidate whether an *EGFR* mutation status affects the diversity of TCR repertoires, we performed next-generation sequencing-based TCRβ repertoire analysis and calculated the diversity index of 39 lung adenocarcinoma samples. In the TCRβ sequencing, we obtained

TABLE 1 Patient characteristics

Characteristics	Total (n = 39)	*EGFR* status		*P*-value
		Mutant (n = 20)	Wild-type (n = 19)	
Age (median, years) (range)	68 (41-79)	68 (41-85)	66 (56-85)	.31
Sex				
Male	14 (36%)	4 (20%)	10 (53%)	**<.05**
Female	25 (64%)	16 (80%)	9 (47%)	
Smoking				
Current/extent	20 (51%)	6 (30%)	14 (74%)	**.01**
Never	19 (49%)	14 (70%)	5 (26%)	
Pathological stage (pStage)				
Stage I	9 (23%)	6 (30%)	3 (16%)	.74
Stage II	16 (41%)	5 (25%)	11 (58%)	
Stage III	14 (36%)	9 (45%)	5 (26%)	

EGFR, epidermal growth factor receptor.
The significant *P*-values are shown in bold ($P < .05$).

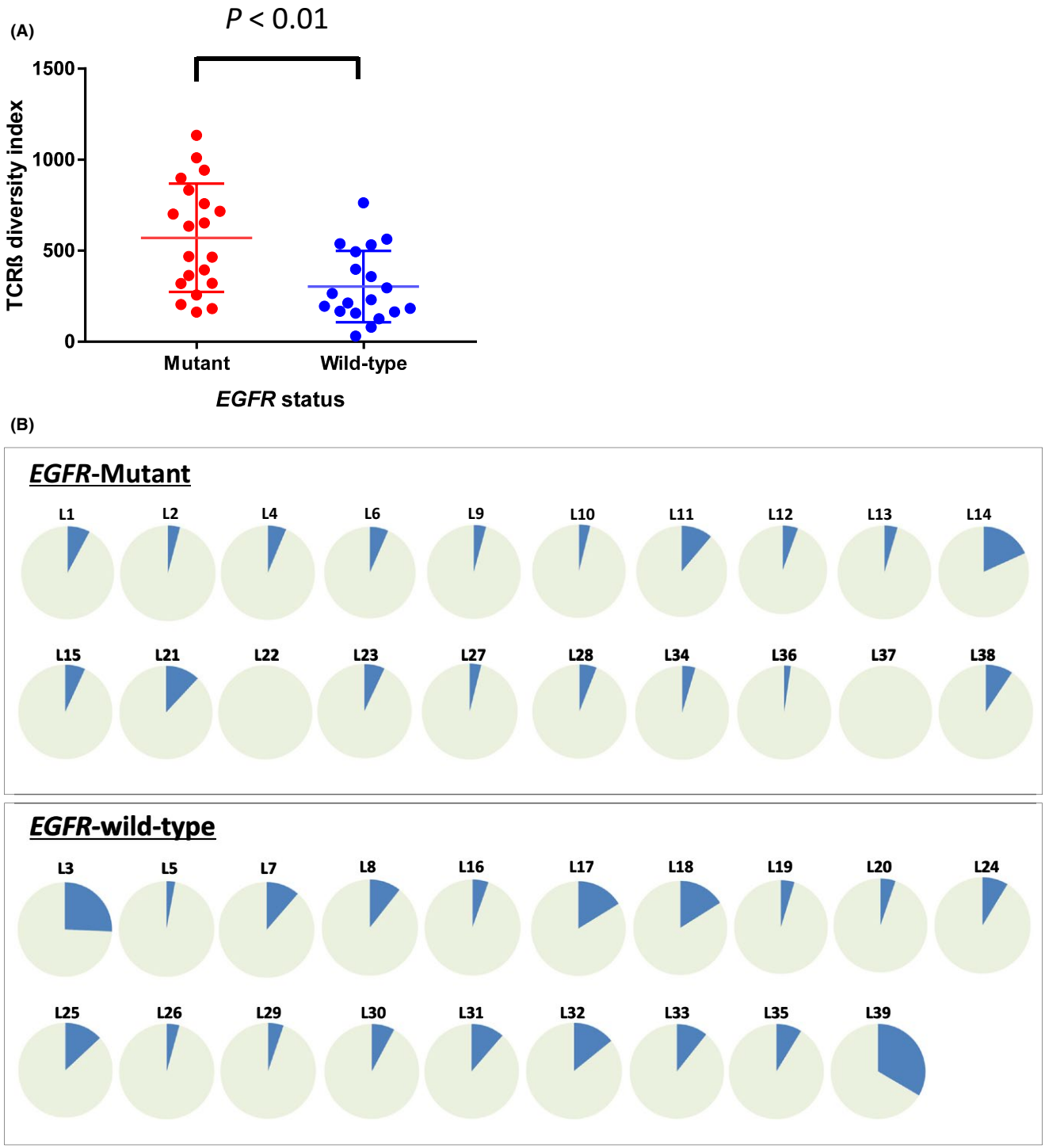

FIGURE 1 TCRβ diversity index (A) and the sum of frequencies of the TCRβ clonotype of 1% or higher *EGFR*-mutant and wild-type tumors (N = 39) (B, C). A, The *EGFR*-mutant tumors had a higher TCRβ diversity index than wild-type tumors (median [range] 552 [162-1135] vs 230 [30-764]; $P < .01$). B, The blue portion of each pie chart shows the cumulative sum of frequencies of the TCRβ clonotype of 1% or higher in *EGFR*-mutant and wild-type tumors. C, The proportions of TCRβ clones in tumors with wild-type *EGFR* were significantly higher than those in tumors with *EGFR* mutations (median [range]: 5.8% [0%-18.2%] vs 10.6% [2.9%-33.4%]; $P = .01$). Number of non-synonymous mutations (D) and predicted neoantigens (E) in *EGFR*-mutant and wild-type tumors ($N = 16$). D, Number of non-synonymous mutations in *EGFR*-mutant and wild-type tumors. The number of non-synonymous mutations was significantly lower in *EGFR*-mutant than wild-type tumors (median [range]: 26 [10-63] vs 87 [28-193]; $P < .01$). E, Number of predicted neoantigens in *EGFR*-mutant and wild-type tumors. *EGFR*-mutant tumors had a significantly lower number of predicted neoantigens than wild-type tumors (median: 57 [4-221] vs 157 [47-247]; $P = .03$). *P*-values were calculated to test the difference between the *EGFR*-mutant and wild-type groups using the unpaired *t* test. EGFR, epidermal growth factor receptor; TCR, T cell receptor

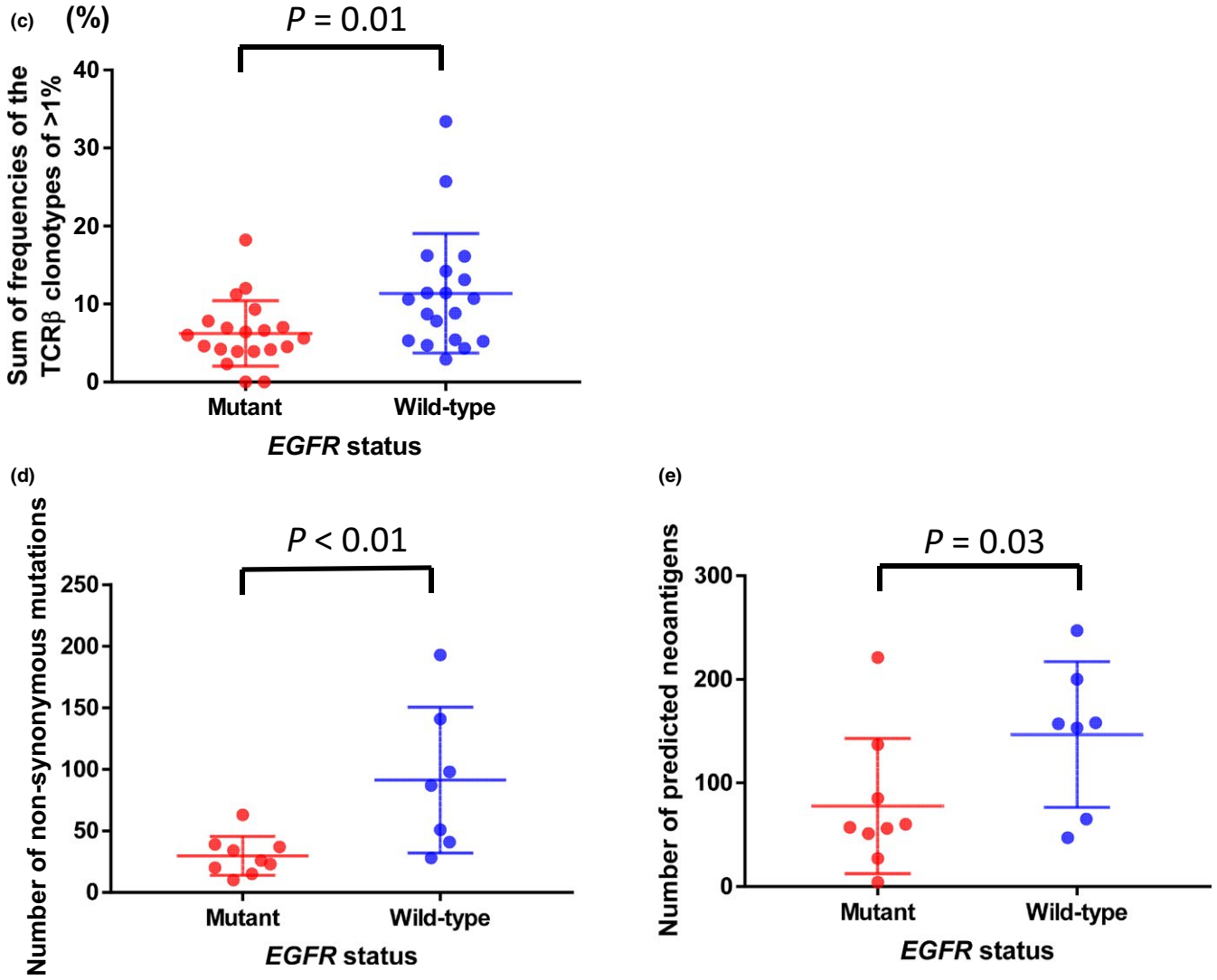

FIGURE 1 (Continued)

a total of 804 134 ± 358 995 sequence reads (average ± SD) mapped to the V, J and C segments, and identified 55 343 ± 32 756 unique CDR3 clonotypes (Table S1). Notably, tumors with *EGFR* mutations had a higher TCRβ diversity index than those without *EGFR* mutations (median [range] 552 [162-1135] vs 230 [30-764]; $P < .01$; Figure 1A, Table S1). Multivariate logistic regression analyses were undertaken to examine whether the correlation between the diversity index and the *EGFR* status would be influenced by differences in patient characteristics such as age, sex, smoking and pathological stage. We found that the TCRβ diversity index (High: greater than median value) and smoking status (non-smoker) were independently associated with *EGFR* mutation status ($P = .04$ and .02, respectively; Table 2).To further examine differences in the proportions of the expanded T cell clones in the 2 groups with and without *EGFR* mutations, we compared the sum of frequencies of the TCRβ clonotypes of 1% or higher in the 2 groups (Figure 1B, Table S1). The sum of frequencies of the TCRβ clonotypes of 1% or higher in tumors with wild-type *EGFR* were significantly higher than those in tumors with *EGFR* mutations (median [range]: 5.8% [0-18.2%] vs 10.6% [2.9-33.4%]; $P = .01$; Figure 1C). The results showed the same tendencies when we used the sum of frequencies of the TCRβ clonotypes of .5% and 2% as cut-off values ($P = .02$, $P < .01$, respectively; Figure S1)

3.3 | Comparison of the numbers of somatic non-synonymous mutations/predicted neoantigens between epidermal growth factor receptor-mutant and wild-type tumors

To assess a relationship between the *EGFR* status and the numbers of somatic non-synonymous mutations, we compared the numbers in tumors with and without *EGFR* mutations. WES analysis was performed for 16 randomly selected cases (Del19/L858R/wild type were 5/4/7, respectively) from the 39 lung adenocarcinomas. We identified a total of 906 somatic non-synonymous mutations (10-193 per individual patients; Table S2). The number of non-synonymous mutations was significantly lower in *EGFR*-mutant tumors than in

TABLE 2 Multivariate logistic regression analyses of variables related to *EGFR* mutation status

Variable	Category	Regression coefficient	Standard error	Odds ratio (95% CI)	*P*-value
Diversity index in tumor	High (>364[a])	1.91	.93	6.76 (1.09-41.80)	**.04**
Age	65 or more	.74	.89	2.10 (.37-11.98)	.40
Sex	Female	1.11	.88	3.04 (.54-17.22)	.20
Smoking	Non-smoker	2.15	.93	8.57 (1.38-53.32)	**.02**
pStage	IIb/III	−.29	.86	.75 (.14-4.07)	.74
Constant		−2.68	1.04	.07	.01

CI, Confidence interval.
[a]Median value.
The significant *P*-values are shown in bold ($P < .05$).

EGFR-wild-type tumors (median [range]: 26 [10-63] vs 87 [28-193]; $P < .01$; Figure 1D). To verify this result, we examined the data of lung adenocarcinomas in the TCGA dataset and found similar results supporting that the mutational burden was lower in the *EGFR*-mutant group than the *EGFR*-wild-type group (median [range]: 48 [16-241] vs 191 [0-1277], $P < .01$; Figure S2).

To further investigate the relation between the numbers of predicted neoantigens and the *EGFR* status, we performed in silico neoantigen prediction for non-synonymous mutations in the 16 lung adenocarcinomas in which we conducted WES. We predicted the binding affinity of peptides including an amino-acid substitution to individual HLA-A, B and C molecules that were estimated from the WES data of normal DNAs. We obtained neoantigen epitope candidate sequences, which were filtered with the binding affinity to the HLA molecules of 500 nM or lower, and identified a total of 469 neoantigen candidates (4-247 neoantigens in individual patients; Table S2). Subsequently, we compared the number of candidate peptides in the 2 groups with and without *EGFR* mutations and found that tumors with *EGFR* mutations had significantly lower numbers of predicted neoantigens than those without *EGFR* mutations (median: 57 [4-221] vs. 157 [47-247]; $P = .03$; Figure 1E).

3.4 | Correlation between the proportion of expanded T cell receptor β clones and the number of non-synonymous mutations

To analyze the relationship between the clonal expansions of T cells in tumor microenvironment and the numbers of non-synonymous mutations, we compared the sum of frequencies of the TCRβ clonotypes of 1% or higher with the number of non-synonymous mutations in the 2 groups with and without *EGFR* mutations (Figure 2). The numbers of non-synonymous mutations were significantly correlated with the clonal T cell expansions in the tumor tissues ($r = .52$, $P = .04$).

3.5 | Immune-related gene expression analysis

Because PD-1/PD-L1 expression is important to immune responses in tumors, including the efficacy to ICI, we assessed PD-1/PD-L1 expression levels in tumors with and without *EGFR* mutations. We measured mRNA expression levels of PD-1 and PD-L1 in 39 lung adenocarcinomas and compared the expression levels between the 2 groups. We observed no significant differences in PD-1 and PD-L1 mRNA expression levels between the *EGFR*-mutant and wild-type groups ($P = .09$, $P = .25$, respectively). We also observed no significant differences in the *CD8/FOXP3* ratio between the 2 groups ($P = .38$). However, we found the tendency of a higher *PD-1/CD8* expression ratio in *EGFR*-wild-type tumors compared to *EGFR*-mutant tumors (median: .10 [.02-.48] vs .13 [.01-2.35]; $P = .24$), implying that *EGFR*-wild-type tumors might have a more immune-active microenvironment.

4 | DISCUSSION

The underlying biology for lower clinical response rates of ICI in lung adenocarcinomas having *EGFR* mutations is not well understood. Hence, identification of predictive biomarkers for responses to ICI is critical for lung cancer patients with *EGFR* mutations. We analyzed immune-related microenvironment in tumors with and without *EGFR* mutations using TCRβ repertoire analysis and WES. Our study is the first to characterize distinct TCR repertoire patterns between 2 groups of lung adenocarcinoma with and without *EGFR* mutations; we also clarified the association between the diversity of TCR repertoires and the mutational load in tumors. Our findings may evoke further understanding of the molecular mechanism through which *EGFR*-mutant patients show poor clinical responses to ICI.

In this study, we demonstrated that the sum of frequencies of the TCRβ clonotypes of 1% or higher in tumors with wild-type *EGFR* were significantly higher than those in tumors with *EGFR* mutations (Figure 1C). Clonal T cell expansion in the tumor microenvironment is essential for the effective anti-tumor immune response. Recent studies suggested that responses to ICI were associated with the clonal expansion of tumor-infiltrating T lymphocytes.[25,26] Therefore, our findings implied that the low clonal T cell expansion in tumors with *EGFR* mutations might be a critical factor related to the unfavorable response to ICI. Furthermore, TCR sequencing might be applicable for the treatment selection in patients with *EGFR* mutations by evaluating the proportions of TCRβ clones in the tumor.

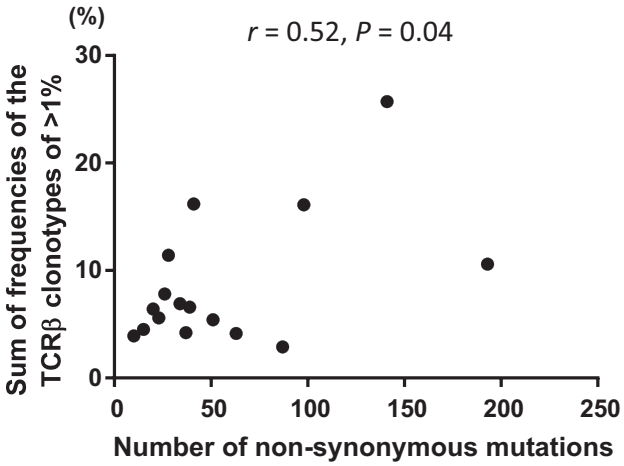

FIGURE 2 Correlation analysis of the number of non-synonymous mutations and the sum of frequencies of the TCRβ clonotype of 1% or higher. The number of non-synonymous mutations was significantly correlated with the sum of frequencies of the TCRβ clonotype of 1% or higher. TCR, T cell receptor

This is the first study showing significant differences in the numbers of neoantigens between lung adenocarcinomas with and without *EGFR* mutations; we demonstrated that tumors with *EGFR* mutations had lower numbers of non-synonymous mutations and predicted neoantigens than those without *EGFR* mutations. Previous studies have reported that the efficacy of antibody targeting PD-1/PD-L1 is associated with the tumor mutation burden (TMB) and the numbers of predicted neoantigens in NSCLC.[27-29] Hence, we assume that the lower TMB may partly explain the lower efficacy of lung cancer with an *EGFR* mutation to ICI.[30]

In addition, patients' characteristic may affect the efficacy of ICI. *EGFR* mutations are found in female patients with no history of tobacco smoking.[31,32] Our study population also showed more women than men in the *EGFR*-mutated group. Interestingly, the pooled analysis showed that a higher benefit of ICI treatment was observed in men than women, regardless of histological type.[33] Furthermore, several studies report that tobacco smoking leads to a higher mutation burden in human cancers.[27,34,35]

PD-L1 expression levels may affect the clinical benefit of ICI. In the combined analysis of 15 reported studies, *EGFR*-mutated tumors showed low PD-L1 expression in tumors[30] and recent studies demonstrated that PD-L1 expression levels were associated with *EGFR* mutation status.[36,37] However, in this study, PD-1/PD-L1 expression levels were not significantly different between the 2 groups with and without *EGFR* mutations. Accumulated data now indicates that the PD-L1 status alone is not a useful biomarker for the prediction of the efficacy of ICI.[38-40]

In conclusion, the present study offers novel evidence that lung adenocarcinoma with *EGFR* mutations have a higher TCRβ diversity index and a lower number of neoantigens compared with tumors without *EGFR* mutations, and could explain impaired responses to ICI. Furthermore, TCR repertoire analysis might provide useful information for identification of good responders for immunotherapy in *EGFR*-mutant NSCLC.

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CONFLICT OF INTEREST

Y. N. is a stock holder and a scientific advisor of OncoTherapy Science. K. K. and S.-K. L. are scientific advisors of Cancer Precision Medicine. No potential conflicts of interest were disclosed by the other authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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